

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

JUL 12 2004

U.S. TRADEMARK OFFICE
REG. U.S. PAT. & T. OFF.

Suppression of Two Cloned Smooth Muscle-Derived Delayed Rectifier Potassium Channels by Cholinergic Agonists and Phorbol Esters

FIVOS VOGALIS, MANUS WARD, and BURTON HOROWITZ

Department of Physiology, University of Nevada, School of Medicine, Reno, Nevada 89557-0046

Received July 11, 1995; Accepted September 5, 1995

SUMMARY

Functional coupling between muscarinic (m_3) receptors and two voltage-gated K^+ (K_v) channels ($K_{v1.2}$ and $K_{v1.5}$) cloned originally from canine colonic smooth muscle was studied using the *Xenopus* oocytes expression system and a mammalian cell line (COS cells). Oocytes were coinjected with cRNAs encoding the human m_3 receptor and the K_v channel clones. COS cells were stably transfected with the hm_3 cDNA and the cDNA encoding $K_{v1.5}$ channels. In oocytes coexpressing hm_3 receptors and K_v channels, acetylcholine (ACh, 100 μM) decreased the whole-oocyte K_v channel current (I_{Kv}) by 72% over 20 min. ACh was equally effective at suppressing $I_{Kv1.2}$ as $I_{Kv1.5}$. In oocytes expressing only K_v channels, phorbol esters (phorbol

dibutyrate) and phorbol didecanoate (10–30 nM) mimicked the action of ACh on I_{Kv} in oocytes coexpressing hm_3 receptors. At the single-channel level, both ACh and phorbol dibutyrate applied to the extra-patch membrane reduced the open probability of K_v channels in the cell-attached patches without affecting single-channel conductance. In cotransfected COS cells, over a similar time course as in oocytes ACh suppressed whole-cell $I_{Kv1.5}$, but only by 30%, and the effect was not reversible. These data indicate that stimulation of m_3 receptors in cells that express $K_{v1.2}$ and $K_{v1.5}$ channels causes a poorly reversible decrease in the open probability of these channels.

Intracellular second messenger pathways constitute an important link between cell-surface receptor stimulation and cellular electrical activity. These signaling pathways target many cellular proteins, including K_v channels (1). Modulation of K_v channels in many cell types is believed to occur through channel phosphorylation by protein kinases, including cAMP-activated kinase (protein kinase A) and PKC (2). K_v channels, including M-channels and K_{del} , are targeted by muscarinic agonists. In rat superior cervical sympathetic neurons, suppression of I_{Km} by muscarinic agonists requires the generation of a diffusible second messenger (3). This process is transduced by $G_{\alpha q}/G_{\alpha 11}$ and involves activation of PLC β (4). Activators of PKC partially suppress I_{Km} in frog sympathetic ganglion cells but inhibitors of PKC have little effect on the suppression of I_{Km} by agonist peptides (5). Suppression of I_{Km} by muscarinic agonists, however, is at-

tenuated by intracellularly applied Ca^{2+} buffers, alluding to mediation by a Ca^{2+} -dependent enzyme such as PKC (6). A direct action of either the activated G_α protein fragment or $G_{\beta\gamma}$, however, cannot be ruled out (3). A I_{Km} -like current has also been reported in toad gastric smooth muscle cells (7), where it underlies much of the cholinergic receptor-mediated excitation. In this tissue, as in sympathetic ganglion cells, activators of PKC suppressed the current, but it was hypothesized that these compounds may have a direct inhibitory action on the channels.

In rat ventromedial hypothalamic neurons, muscarinic receptor stimulation suppresses a K^+ current with properties consistent with a I_{Kdel} channel current, through activation of $G_{\alpha 11}$ (8). The steps, if any, subsequent to activation of the G protein were not investigated. More recently, modulation of K_v channels by second messenger pathways has been studied using cloned channels. These studies have the advantage over experiments performed in intact cells by using a channel of known primary structure that can be expressed in abun-

This work was supported by National Institutes of Diabetes and Digestive and Kidney Diseases Program Project Grant DK41315.

ABBREVIATIONS: K_v , voltage-gated potassium; I_{Kv} , voltage-gated potassium current; I_{Km} , potassium M-current; PKC, lipid- and Ca^{2+} -activated kinase (protein kinase C); ACh, acetylcholine; hm_3 , human muscarinic m_3 receptor; K_{del} , delayed rectifier K^+ channel; I_{Kdel} , delayed rectifier K^+ channel current; I_{Ca-Cl} , Ca^{2+} activated chloride current; $G_{K_{max}}$, maximal cellular K^+ conductance; $V_{0.5}$, voltage of half-maximal availability; PLC, phosphokinase C; DMSO, dimethylsulfoxide; PDBu, phorbol dibutyrate; PDD, phorbol didecanoate; IP₃, inositol-1,4,5-triphosphate; I-V, current-voltage; PTX, pertussis toxin; PIP₂, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CSA, capacitative surface area; $[Ca^{2+}]_{free}$, free Ca^{2+} concentration; $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

dance, to the exclusion of other contaminant K^+ channels and other ion channels. These studies have shown that a number of K_v channels are potently modulated by second messenger systems, including the pathway linking G protein-coupled receptors and PKC (9).

We have cloned two delayed-rectifier type K^+ channels ($K_{v}1.2$ and $K_{v}1.5$) from the circular muscle layer of the canine colon (10, 11). These channels, probably as heteromultimers (12), may contribute to a large portion of the $I_{K\text{del}}$ recorded from native myocytes (13). $I_{K\text{del}}$ is activated during membrane depolarization and contributes to the maintenance of the "slow wave" plateau potential (14). In the canine colon, muscarinic receptor stimulation enhances excitatory electrical and mechanical activity (15) by increasing the amplitude and duration of slow waves. Colonic myocytes express both m2 and m3 receptor subtypes (16), but only the m3 receptor is coupled to the PLC pathway (17). Because $I_{K\text{del}}$ is activated during the plateau potential, it is a reasonable hypothesis that these channels may be an important target of cholinergic agonists in colonic smooth muscle. We have been unable to unequivocally demonstrate a specific action of cholinergic agonists on $I_{K\text{del}}$ in colonic smooth muscle cells. Pharmacological interventions to eliminate contaminating Ca^{2+} -activated K^+ currents with externally applied tetraethyl ammonium ions may interfere with agonist binding to muscarinic receptors, and inorganic (Cd^{2+} , Ni^{2+}) blockers of L-type Ca^{2+} channels may interfere with the voltage-dependent gating of K_{del} channels (18). Furthermore, ACh activates a large non-selective cation current in colonic smooth muscle cells in a voltage range that overlaps with the activation range of $I_{K\text{del}}$ (19). Although these impediments to analysis of cholinergic effects on $I_{K\text{del}}$ are not insurmountable, we decided to first approach the problem using cloned channels known to underlie this current. We coexpressed the two cloned K^+ channels in *Xenopus* oocytes and in COS cells with hm3. These expression systems make it possible to study the coupling between m3 receptor stimulation and modulation of K_{del} channels.

The results presented in this report indicate that the opening of both $K_{v}1.2$ and $K_{v}1.5$ channels can be suppressed by activation of a second messenger pathway linked to the m3 receptor. This pathway is coupled through a G protein and requires, as a prerequisite, a rise in intracellular Ca^{2+} levels. The roles of PKC and other possible second messengers are discussed.

Experimental Procedures

Oocyte injection. Ovarian lobes were removed from anesthetized adult female *Xenopus laevis* frogs (*Xenopus* 1, Ann Arbor, MI) under sterile conditions. The lobes were then mechanically opened, and the oocyte follicular layer was removed by incubation with collagenase (1 mg/ml) in Ca^{2+} -free ND96 solution at room temperature for 2–3 hr. The oocytes were then collected, rinsed, and stored in ND96 solution containing (in mM) 2.5 pyruvate, 96 NaCl, 1.5 $CaCl_2$, 2 KCl, 1 $MgCl_2$, and 5 HEPES plus antibiotics (100 units/ml gentamycin) at 19° for up to 24 hr before injection. cRNA encoding $K_{v}1.2$ and $K_{v}1.5$ channels and hm3 receptor was injected in a total volume of 50 nl, and the oocytes were stored until assay for 2–5 days.

Two-electrode voltage-clamp. Whole-oocyte potassium currents were recorded using the two-electrode voltage-clamp amplifier (Geneclamp 500, Axon Instruments, Burlingame, CA). Voltage-clamp protocols, data acquisition, and analysis were performed by

using pClamp 6.01 software (Axon Instruments). Microelectrodes were drawn to have resistances of 1–2 M Ω when filled with 3 M K-aspartate. Recordings were performed at room temperature (22–25°) in modified ND96 solution in which NaCl was replaced equimolar Na-isethionate to reduce contamination by endogenous Ca^{2+} -activated Cl^- channel currents. Current recordings were not corrected for capacitance and leak currents. Capacitative surface area was calculated by integrating the area under the capacitance current elicited in response to a 10-mV step depolarization from the holding potential (-80 mV), dividing by the magnitude of the voltage step and by the specific membrane capacitance of 1 $\mu F\text{ cm}^{-2}$.

Single-channel recordings. Unitary currents were recorded from cell-attached patches from oocytes mechanically denuded of their vitelline membranes. Removal of the vitelline membrane was performed under a binocular microscope, in most cases without treatment of the oocyte with hypertonic shrinking solution. In experiments of the effect of hm3 receptor stimulation on unitary K_v channel currents, oocytes were bathed in a high- K^+ solution (140 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH titrated to 7.4 with NaOH). The pipette solution contained 135 mM NaCl, 2 mM $MgCl_2$, 2 mM KCl, and 10 mM HEPES, pH titrated to 7.4 with NaOH. In a series of experiments designed to study the effect of $[Ca^{2+}]$ on K_v channel currents in excised inside-out patches, the bathing solution contained 100 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, and 1 mM EGTA. Ca^{2+} was added to this solution to obtain the desired $[Ca^{2+}]_{\text{free}}$ from a 100 mM stock solution (Orion). pH was titrated to 7.4 using KOH. $[Ca^{2+}]_{\text{free}}$ was calculated using the EQCAL program (Biosoft, Ferguson, MO). Patch-pipettes were drawn from thick-walled borosilicate capillary tubing (Sutter BF-150-117-86) on a programmable micropipette puller (Sutter Instruments, Novato, CA). Their tips were fire-polished and coated with Sylgard (Dow Corning) to within 0.5 mm of the tip to reduce electrical noise and capacitance current artifact. Their resistances ranged from 5 to 10 M Ω . Unitary currents were recorded using the patch-clamp circuit of the Geneclamp 500 amplifier equipped with a 100-G Ω resistive headstage. Recordings were low-pass filtered at either 500 or 1000 Hz and digitized on-line at 5 KHz using a DIGIDATA 1200 A/D converter (Axon Instruments). Data acquisition and analysis were performed using pClamp 6.01 software (Axon Instruments). Open probability (NP_o) was calculated by fitting gaussian functions to all-point histograms of the sampled data and applying the following equation: $NP_o = \sum [\sum (a_i)] \cdot n$, where a_i is the proportion of the total area of the distribution occupied by openings to the i th conductance level, and n is the fully open channel level.

Current recording from COS cells. Whole-cell current recordings from COS cells were performed in an electrophysiological chamber positioned on the stage of an inverted microscope (Nikon) and using either conventional whole-cell recording techniques (20) or the perforated patch technique (21). Pipettes were drawn from capillary glass (Sutter BF-150-117-115) and had resistances of 2–5 M Ω when filled with intracellular pipette solution. Suspensions of COS cells stably cotransfected with the hm3 and $K_{v}1.5$ cDNAs were placed on the recording chamber and allowed to adhere to the bottom. The bathing solution contained (in mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 dextrose. pH was adjusted to 7.35 with NaOH. The pipette solution contained (in mM) 140 KCl, 1 $MgCl_2$, 10 HEPES, and 5 BAPTA. Amphotericin-B was made up as a stock solution in DMSO (3 mg/50 ml). Then, 4- μl aliquots of stock solution were dissolved in 1 ml of pipette solution by sonication. Pipettes were filled with this amphotericin-containing solution. After formation of gigaseals, access resistance was monitored using the capacitance and resistance subtraction circuitry of an Axopatch 1C amplifier (Axon Instruments). Adequate access resistance was achieved when access resistance had fallen below 20 M Ω and the current amplitude elicited by constant depolarizing test pulses had stabilized. The time required to achieve these conditions varied from 10 to 35 min after gigaseal formation. Whole-cell currents were low-pass filtered at 1 KHz and digitized at 2.5 KHz using pClamp 5.5 software (Axon

Instruments). Recordings were obtained at room temperature. Cells were perfused continuously at a rate of 1 ml/min. Drugs were added to the perfusing solution. ACh (10^{-1} M) and atropine (10^{-3} M) were made up as stock solutions in distilled water.

Drugs and statistical analysis. The following compounds were used: ACh, carbachol, bethanechol, atropine, pertussis toxin (Sigma Chemical Co., St. Louis, MO). Ionomycin, A23187, PDBu, PDD, 4 α -PDD, H7, Calphostin C, staurosporine, (Calbiochem). Ionomycin was dissolved in pure DMSO (Sigma Chemical Co.) in a stock solution of 10^{-3} M. The phorbol esters were also dissolved in DMSO in stock solution of 10^{-2} M. Calphostin C was dissolved in DMSO in a stock solution of 10^{-3} M and was stored protected from light. The remaining compounds were all dissolved in distilled water. Stock solutions of ACh (10^{-1} M) were stored frozen until use. In preliminary experiments, DMSO inhibited K⁺ channels currents in oocytes at concentrations of $>10^{-4}$ M. The highest concentration of DMSO in our test solution as vehicle did not exceed 10^{-6} M. Where indicated, oocytes were injected with 50 nl of 100 mM EGTA stock solution, pH 7.4, 10 min before current recording. Estimated concentration of EGTA in the cytoplasm was 4.7 mM assuming an oocyte volume of 1 μ l. Where appropriate, Student's *t* test was applied to calculate the statistical significance of differences between mean values, where the number in parentheses represents the number of cells.

Results

Muscarinic (m3) receptor stimulation suppresses I_{Kv} in *Xenopus* oocytes. Before investigating the effects of muscarinic receptor stimulation on I_{Kv}, we tested the expression of the hm3 receptor from the encoding cDNA (provided by Dr. Peralta, Harvard University) (22) by noting the presence of I_{Ca-Cl} in response to superfusion with ACh (ACh, 100 μ M). Oocytes were voltage-clamped at a holding potential of -80 mV. In Cl⁻-containing ND96 solution, I_{Ca-Cl} appeared as a transient (<2 min) increase in inward current at the holding potential and as an increase in the net outward current at potentials positive to 0 mV. This transient nature of the response most probably reflected the depletion of Ca²⁺ from IP₃-sensitive Ca²⁺ stores (17). In oocytes not injected with the hm3 cRNA, ACh failed to activate I_{Ca-Cl}. In the experiments that followed, I_{Ca-Cl} was eliminated by using Cl⁻-deficient ND96 and pipettes filled with 3 M K-aspartate.

ACh, carbachol, and bethanechol all suppressed I_{Kv} at concentrations of >50 μ M. Because the level of expression of the hm3 receptors varied among oocytes, the relative efficacies of these agonists were not tested. For the remainder of the study, we used ACh at a standard concentration of 100 μ M. Continuous superfusion of ACh caused a large reduction in I_{Kv1.2} (Fig. 1A). This reduction of current usually required 15–20 min to reach steady state (Fig. 1C). ACh produced similar effects on K⁺ currents in oocytes expressing K_v1.5 channels (Fig. 1B). The ACh-mediated suppression of I_{Kv} was blocked or severely reduced by pretreatment (10 min) with atropine (1 μ M) (Fig. 1D). Comparison of the I-V relationships for both I_{Kv1.2} and I_{Kv1.5} before and after ACh treatment (Fig. 2, A and B, respectively) revealed that ACh caused an equipotent reduction in currents at all potentials for both clones. These reductions in current were attributable in large part to decreases in the G_{K_{max}} generated by the two K⁺ channel clones. In oocytes expressing K_v1.2 channels, G_{K_{max}} calculated from the steady state availability of I_{Kv1.2} was reduced from 55 to 28 μ S (two); in oocytes expressing K_v1.5 channels, G_{K_{max}} was reduced from 128 to 29.7 μ S (two). The V_{0.5} after ACh treatment were either not affected appreciably (I_{Kv1.2}:

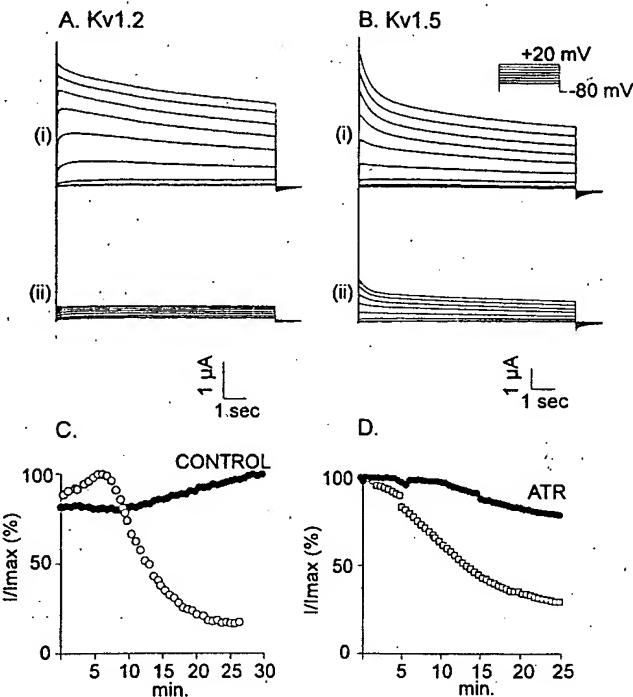


Fig. 1. Effect of ACh (100 μ M) on whole-oocyte K⁺ currents. A, Oocyte coinjected with hm3 receptor and K_v1.2 channel cRNAs. *i*, Oocyte was depolarized from a holding potential of -80 mV to test potentials ranging from -50 to +20 mV in 10-mV increments. I_{Kv1.2} activated rapidly and decayed slowly. *ii*, At 20 min after application of ACh, I_{Kv1.2} was decreased at all test potentials. B, Oocyte coinjected with hm3 receptor and K_v1.5 channel cRNAs. *i*, Currents elicited as in A, in the absence of ACh. Note more rapid inactivation of I_{Kv1.5}. *ii*, I_{Kv1.5} elicited in the presence of ACh. ACh was added 20 min before eliciting currents. C, Time course of ACh-mediated suppression of I_{Kv1.2} elicited by test depolarization to +20 mV (○). ACh was applied 2 min after beginning of recording period. The initial increase in current was not a consistent observation and was due to activation of Ca²⁺-activated Cl⁻ current. In the absence of ACh, I_{Kv1.2} did not decrease with time and sometimes "ran up" (●). D, Effect of atropine (1 μ M) pretreatment (■) on suppression of I_{Kv1.5} by ACh (□).

V_{0.5} control = -16 mV, V_{0.5} ACh = -21 mV [two]) or shifted slightly negative (I_{Kv1.5}; V_{0.5} control = -32 mV, V_{0.5} ACh = -39 mV, [two]) (Fig. 2, C and D). Because of the small magnitude of I_{Kv} after ACh, the plots describing the steady state availability of the currents appeared distorted, making it difficult to accurately measure the steady state parameters (Fig. 2C). In oocytes not expressing the hm3 receptor but only K_v1.2, K_v1.5, or heterotetrameric K_v1.2/K_v1.5 channels, the corresponding currents were not decreased by ACh (Fig. 3). The level of down-regulation of K_v channel currents by ACh treatment may have been underestimated because of possible concomitant activation of endogenous Ca²⁺-dependent cation channels.

m3 receptors, second messenger pathways, and K_v channels. In cells that express m3 receptors, ACh activates PLC_β through a PTX-insensitive G protein (23). PLC_β breaks down PIP₂ to IP₃ and DAG. In turn, DAG may activate PKC, leading to phosphorylation of cellular proteins. We examined the participation of this pathway in the ACh-mediated suppression of I_{Kv} in oocytes by testing the suppression of current to a number of putative inhibitors of PKC. Oocytes coexpressing K_v1.2 channels and m3 receptors were pretreated with staurosporine (0.5 μ M) overnight and then chal-

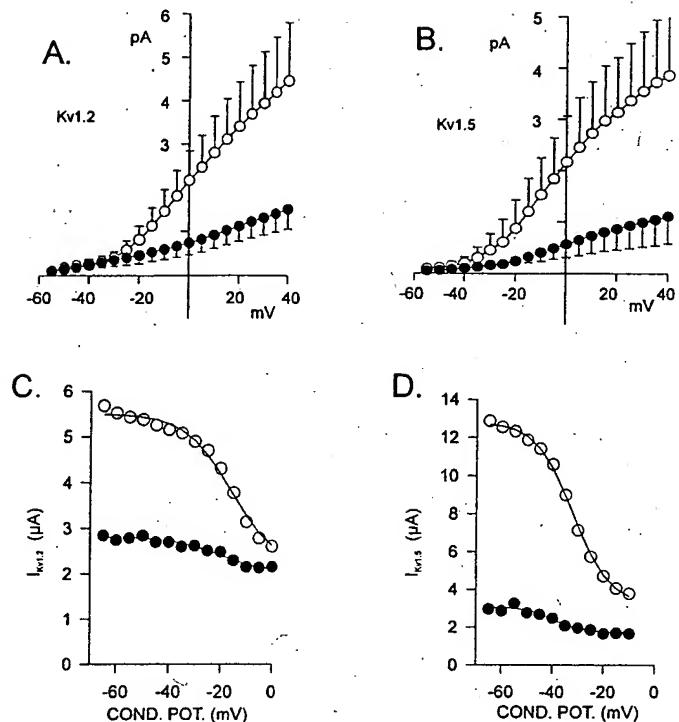


Fig. 2. Effect of ACh on I-V relationships and steady state inactivation of $I_{Kv1.2}$ and $I_{Kv1.5}$. **A.**, I-V relationship for $I_{Kv1.2}$ before (○) and 20 min after (●) application of ACh 100 μM (five). **B.**, I-V relationship for $I_{Kv1.5}$ before (○) and 20 min after (●) application of ACh 100 μM (eight). **C.**, Steady state inactivation of $I_{Kv1.2}$ (control, ○; ACh, ●). $V_{0.5}$ was shifted negative by ACh from -16 to -21 mV (two). **D.**, Steady state inactivation of $I_{Kv1.5}$ (control, ○; ACh, ●). $V_{0.5}$ was also shifted negative by ACh from -32 to -39 mV (two). Steady state inactivation was studied using 15-sec conditioning steps to potentials ranging from -70 to +20 mV, followed by a 200-msec test pulse to +40 mV. The test currents elicited by the pulses to +40 mV were averaged and plotted as a function of conditioning potential. The data points were fitted with a Boltzmann function: $G = (I_{\text{test}} - C)/(1 + \exp[(V - V_{0.5})/V_s]) + C$, where I_{test} is the peak current elicited by the test pulse, V is the conditioning voltage, $V_{0.5}$ is the voltage of half-maximal inactivation, V_s is the slope factor, and C is the magnitude of the noninactivating current component.

lenged with ACh. Although staurosporine pretreatment failed to fully block the ACh-mediated suppression of $I_{Kv1.2}$, the level of suppression was significantly decreased compared with oocytes not treated with staurosporine ($40 \pm 3\%$ [eight], $p < 0.05$). In another series of experiments, pretreatment of oocytes for 15 min with H7 (10 μM), an unrelated inhibitor of several protein kinases including PKC, also significantly attenuated the ACh-mediated suppression of $I_{Kv1.2}$ ($I_{Kv1.2, \text{H7}}/I_{Kv1.2, \text{control}} = 43 \pm 10\%$ [five], $p < 0.05$). These data suggest that the reduction in $I_{Kv1.2}$ in response to m3 receptor stimulation may have been mediated in part by activation of PKC. However, H7 and staurosporine are nonspecific kinase inhibitors, and the muscarinic response may involve kinases other than PKC.

There are at least two Ca^{2+} - and phospholipid-dependent PKC isoforms in *Xenopus* oocytes (24). Injection of oocytes coexpressing hm3 receptors and $K_v1.2$ channels with EGTA (100 mM, pH 7.4 in the pipette) attenuated the suppression of I_{Kv} in response to ACh. The peak amplitude of $I_{Kv1.2}$ in response to a test depolarization to +40 mV 20 min after application of ACh averaged $52 \pm 7\%$ of control (four). Buffering of $[\text{Ca}^{2+}]$ below basal levels by injection of EGTA did

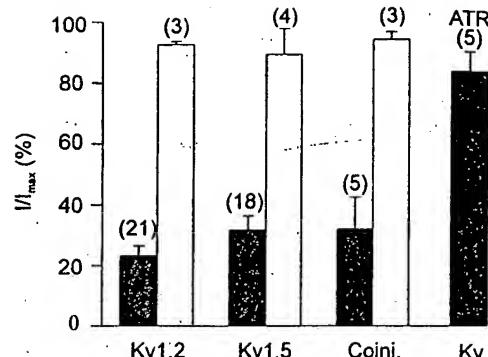


Fig. 3. Effect of ACh on K_v channel currents (mean data). In oocytes coinjected with hm3 receptor cRNA and either $K_v1.2$ or $K_v1.5$ or a mixture of $K_v1.2$ and $K_v1.5$ cRNA, ACh (100 μM) caused a large reduction in whole-oocyte K_v ($K_v1.2$, $K_v1.5$, and $K_v1.2/K_v1.5$) channel currents (filled bars). In oocytes not injected with the hm3 receptor, ACh caused a negligible reduction in I_{Kv} (open bars). Pretreatment of oocytes with atropine (1 μM) markedly attenuated the ACh-mediated reduction in I_{Kv} . Numbers above bars, number of oocytes tested in each group.

not in itself block the channels. Therefore, basal Ca^{2+} is not required to maintain channel activity. These data indicate that the second messenger pathway activated by ACh requires intracellular Ca^{2+} for full activity. Superfusion of Ca^{2+} -free bathing solution after suppression of I_{Kv} by ACh, however, failed to quickly (<25 min) reverse this suppression. I_{Kv} recovered very slowly on superfusion of ACh-free solution (<50% of the current recovered in 40 min). This suggests that the Ca^{2+} -dependent pathway involved in the m3 receptor-mediated suppression of I_{Kv} requires Ca^{2+} for its activation but not for its maintenance. Ca^{2+} dependence could also be demonstrated by application of the Ca^{2+} ionophores A23187 (1 μM) (one) and ionomycin (0.1 μM) (three). Both compounds suppressed I_{Kv} by 50% and by $46 \pm 11\%$, respectively, on a time scale identical to the action of ACh.

The G protein that transduces the ACh-mediated suppression of I_{Kv} in oocytes appears to be PTX sensitive. Pretreatment of oocytes with PTX (2 $\mu\text{g}/\text{ml}$) overnight attenuated the ACh-mediated suppression of $I_{Kv1.2}$. $I_{Kv1.2}$ was reduced to $61 \pm 4.8\%$ of control by ACh in five PTX-treated oocytes compared with only $13 \pm 4.3\%$ in three oocytes from the same batch not treated with PTX ($p < 0.05$). Because m3 receptors are believed to be exclusively coupled to the PTX-insensitive $G_{\alpha q}/G_{\alpha 11}$ class of G proteins (25), our data suggest that the G protein coupled to the m3 receptor in oocytes is atypical or that a combination of PTX-sensitive and -insensitive G proteins is involved (26).

The reduction in I_{Kv} by ACh was not associated with loss of membrane surface area in oocytes. In oocytes coexpressing hm3 receptors and $K_v1.2$ channels, CSA averaged $0.18 \pm .01 \text{ cm}^2$ (seven) before ACh and $0.17 \pm .03 \text{ cm}^2$ after ACh (seven). CSA was examined because a loss of surface membrane may have resulted from internalization of m3 receptors and colocalized K_v channels in the membrane, after phosphorylation of the receptors by intracellular kinases related to β -adrenergic receptor kinase (27).

Effect of phorbol esters on I_{Kv} . Phorbol esters activate PKC directly by binding to the regulatory site on the enzyme (34). We tested the actions of three phorbol ester analogues on whole-oocyte currents generated by the opening of homo-

meric K_v1.2, K_v1.5, and heterotetrameric K_v1.2/K_v1.5 channels. PDBu (30 nM) (Fig. 4, A and B) and PDD (10 nM) each caused marked suppression of all three currents expressed in oocytes ($I_{Kv\text{ PDBu}}/I_{Kv}$, $32 \pm 7.4\%$ [eight]; $I_{Kv\text{ PDD}}/I_{Kv}$, $32 \pm 11\%$ [three]). Maximal suppression of I_{Kv} occurred after 20 min of application, over which time these currents normally show little rundown (Fig. 4E). The slopes of the I-V relationships of both $I_{Kv1.2}$ and $I_{Kv1.5}$ were decreased by PDBu (10 nM) (Fig. 4, C and D). The inactive phorbol ester analogue 4 α -PDD (10 nM), which does not activate PKC, produced only a small reduction in I_{Kv} ($I_{Kv\text{ 4}\alpha\text{-PDD}}/I_{Kv}$, $82 \pm 4\%$ [seven]) (Fig. 4F). Calphostin C (0.1 μ M), a specific PKC inhibitor that binds to the regulatory site on the enzyme, reduced $I_{Kv1.2}$ by 29% (three) alone but did not attenuate the PDBu (10^{-8} M)-mediated suppression of this current: $I_{Kv1.2}$ was decreased to 25% of control (three). This suggests that calphostin C may have an intrinsic PKC-activating action or that it may directly block K_v channels. Staurosporine (0.5 μ M overnight), a more potent PKC inhibitor, significantly attenuated PDBu-mediated suppression of I_{Kv} ($p < 0.05$ unpaired t test; $I_{Kv\text{ PDBu stauroporine}}/I_{Kv\text{ stauroporine}}$, $66 \pm 8\%$ [six]). This sug-

gests that PKC partially mediated the inhibitory action of phorbol ester on K_v channels. PDBu (30 nM) did not significantly effect $V_{0.5}$ of $I_{Kv1.5}$ ($p > 0.05$; $V_{0.5\text{ control}} = -36 \pm 7$ mV; $V_{0.5\text{ PDBu}} = -40 \pm 7$ mV [four]) but decreased maximal conductance from 54.8 ± 8 to 19 ± 8.9 μ S, suggesting that either the number of activatable channels or the single-channel conductance was reduced by PDBu treatment. CSA was unaffected by PDBu (10–30 nM: CSA_{control}, 0.20 ± 0.04 cm^2 ; CSA_{PDBu}, 0.21 ± 0.04 cm^2 [eight]). Other investigators have concluded that internalization became significant only at concentrations of PDBu of >88 nM and at 10 nM PMA (28). The highest PDBu concentration used in the present study was 30 nM. However, slow internalization of channels without any detectable decrease in cell surface area is a possibility that must be mentioned.

Effect of ACh on unitary K_v channel currents. To distinguish between an effect of ACh on channel availability and channel conductance, we obtained single-channel recordings from cell-attached patches in oocytes coexpressing K_v channels and m3 receptors. Results from one such experiment are illustrated in Fig. 5. The patch contained at least two channels that opened on depolarization of the patch from a holding potential of -80 mV to a test potential of 0 mV. Under an asymmetrical [K⁺] gradient ($[K^+]_{\text{pipette}}/[K^+]_{\text{bath}} = 2:140$ mM) test depolarization to 0 mV eliminated nonselective cation channel currents through the ubiquitous stretch-activated channels and allowed for the selective recording of K_v1.2 unitary channel currents. In the absence of ACh, test depolarizations to 0 mV elicited channel openings from at least two K_v1.2 channels (Fig. 5A). An all-points histogram plot of the events recorded during the test potential yielded an NP_o of 1.069 (Fig. 5B). Whole-oocyte $I_{Kv1.2}$ was also recorded from the same oocyte using the two-electrode voltage-clamp in response to a step depolarization to $+40$ mV from a holding potential of -80 mV to $+40$ mV (Fig. 5F). On the application of ACh (100 μ M), $I_{Kv1.2}$ decreased significantly over 15 min (Fig. 5F). Depolarization of the cell-attached patch on the same oocyte after suppression of $I_{Kv1.2}$ now activated fewer channel openings (Fig. 5C). The majority of test pulses failed to activate any channel openings. An all-points histogram plot of these recordings confirmed that the unitary current was unaffected but NP_o was substantially decreased to 0.026 (Fig. 5D). The ensemble-averaged currents before and after application of ACh revealed a similar degree of reduction as the whole-oocyte current (Fig. 5E). These recordings show clearly that m3 receptor stimulation decreases the availability of K_v1.2 channels but does not affect channel conductance. In seven of nine cell-attached patches containing K_v1.2 channels, NP_o was reduced from 1.138 ± 0.18 to 0.55 ± 0.14 , 15–20 min after application of ACh to the cell membrane outside the patch. The unitary current measured from all-point histogram plots averaged 0.79 ± 0.06 pA before ACh and 0.76 ± 0.05 pA after ACh. In a similar manner, in three of five cell-attached patches from oocytes expressing K_v1.5 channels, the NP_o was reduced from 0.603 ± 0.23 to 0.126 ± 0.08 after application of ACh to the bathing solution.

Effect of PDBu on unitary K_v channel currents. To test that PDBu did not affect single-channel conductance, we recorded K_v1.2 channel activity in two cell-attached patches before and after application of PDBu (30 nM) to the membrane outside the patch. Recordings and data from one such

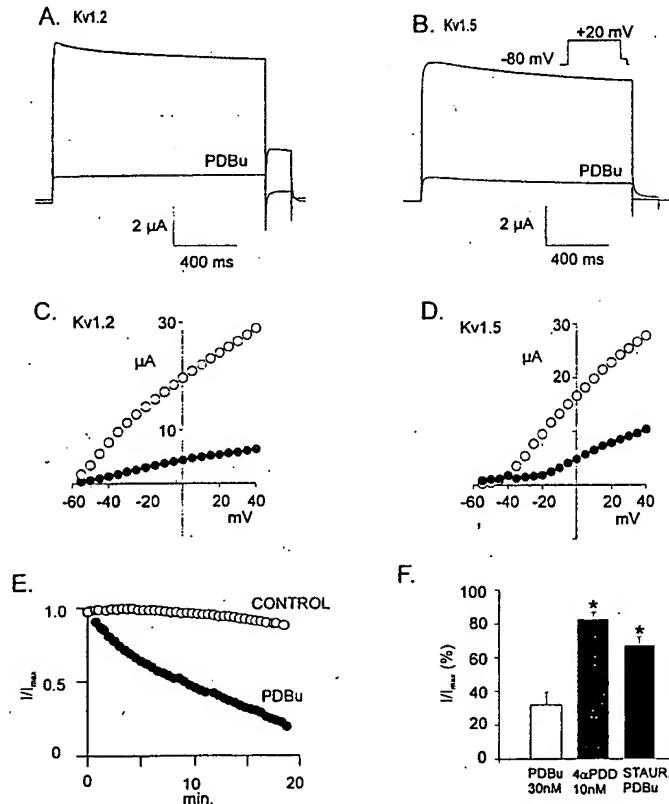


Fig. 4. Suppression by PDBu of whole-oocyte K_v channel currents. A, 30 nM PDBu decreased $I_{Kv1.2}$ elicited by depolarization of the oocyte from a holding potential of -80 mV to a test potential of $+20$ mV. B, 30 nM PDBu also reduced whole-oocyte $I_{Kv1.5}$ by $>80\%$ of control current. C, Average I-V relationship of $I_{Kv1.2}$ before and after application of PDBu (10 nM) (two). D, I-V relationship of $I_{Kv1.5}$ before and after application of PDBu (10 nM) (two). E, Typical time course of reduction of $I_{Kv1.5}$ by PDBu (●) compared with the current elicited in an untreated oocyte (○). F, Average data. PDBu (30 nM) decreased I_{Kv} (calculated from pooled data; open bar [five]) significantly more than the inactive 4 α -analogue of PDD (filled bar [seven]) indicated with * ($p < 0.05$). Overnight pretreatment with staurosporine (0.5 μ M) significantly attenuated the PDBu-mediated reduction in I_{Kv} (six). *, Significant difference from PDBu alone (open bar).

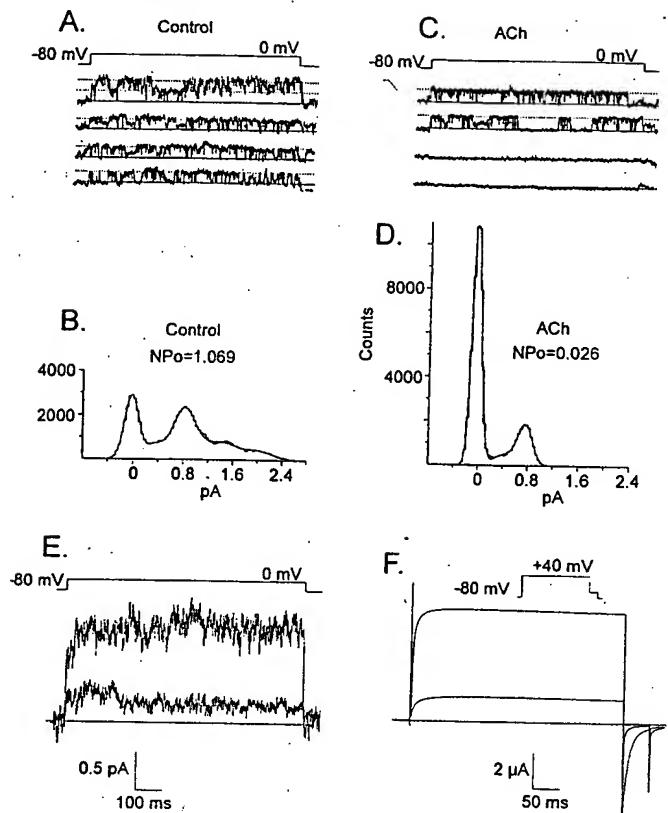


Fig. 5. Muscarinic (m3) receptor stimulation decreases the open probability of $K_v1.2$ channels via a diffusible intracellular second messenger. The oocyte was coinjected with hm3 and $K_v1.2$ cRNAs. **A.** Cell-attached patch recording. Oocyte was bathed in high- K^+ solution (140 mM). Pipette solution contained 2 mM K^+ . The patch was depolarized from a holding potential of -80 mV to a test potential of 0 mV for 900 msec every 8 sec, which evoked opening of $K_v1.2$ channels after a short latency. At least two channels are evident in this patch. **B.** All-points histograms constructed from the channel activity recorded at 0 mV. Unitary current was estimated as 0.8 pA, and NP_o was estimated from the areas under the fitted gaussian curves as 1.069 . **C.** 20 min after application of ACh $100\ \mu M$ to the membrane outside the patch, depolarization to 0 mV elicited fewer channel openings. Four episodes containing channel openings are shown. The majority of the 20 episodes contained no channel openings. **D.** All-points histogram 1 of events at 0 mV after application of ACh. Note that unitary current is unchanged, but NP_o was decreased to 0.026 . **E.** Ensemble averaged currents from 20 consecutive episodes as depicted in **A** and **C**, before and after application of ACh. **F.** Whole-oocyte currents recorded from the same oocyte before and 16 min after application of ACh. Test potential, $+40$ mV. Note parallel reduction in whole-oocyte current and ensemble averaged patch current shown in **E**.

experiment are shown in Fig. 6. Because this patch contained up to four channels, unitary openings were discerned by adjusting the holding potential to -50 mV and depolarizing the patch to 0 mV (Fig. 6A). At least two subconductance states were apparent. As in Fig. 5, B and D, NP_o was estimated by fitting a sum of four gaussian functions to the all-points histogram plot of the events elicited during test pulses (Fig. 6B). The conductance levels of the open channel are indicated by the arrows. Washin of PDBu ($30\ nM$) was monitored by simultaneously recording whole-oocyte current (Fig. 6E). Whole-oocyte $I_{Kv1.2}$ was decreased by $>80\%$ over 15 min (Fig. 6F). After this period, depolarization of the cell-attached patch from -50 to 0 mV failed to activate any channel openings. By increasing the holding potential to -80 mV, however, depolarization of the patch to 0 mV activated

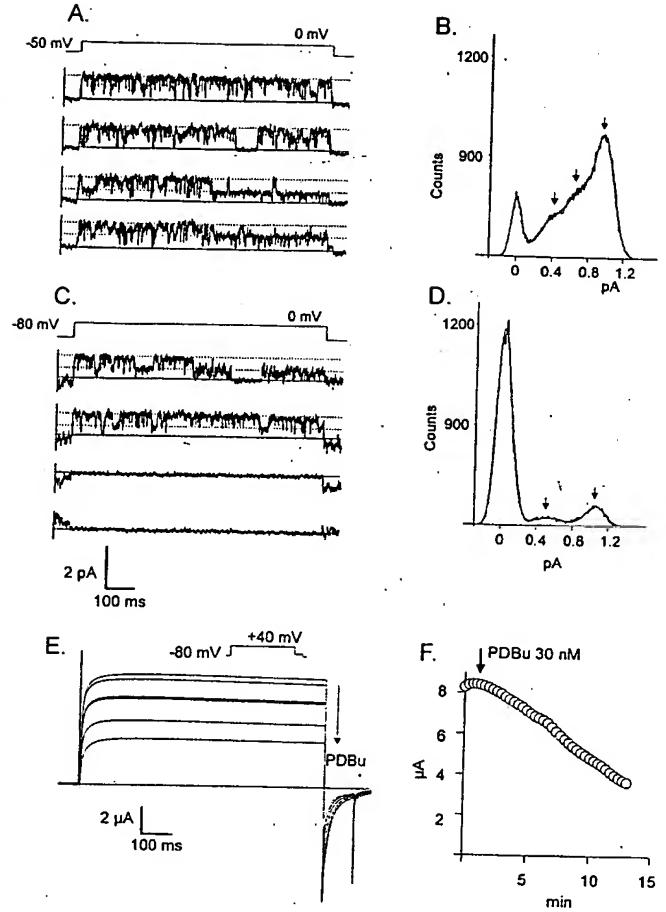


Fig. 6. Extra-patch application of PDBu decreases the open probability of $K_v1.2$ channels. **A.** Cell-attached recording as in Fig. 4A. Unitary channel openings were elicited by adjusting the holding potential (V_h) to -50 mV and applying depolarizing steps to 0 mV every 8 sec. **B.** All-points histogram of events at 0 mV. Note multiple subconductance states in this channel. $NP_o = 0.892$. **C.** At 20 min after application of PDBu $30\ nM$ to the cell membrane outside the patch, depolarization of the patch to 0 mV elicited no openings. However, some channel openings could be elicited by increasing V_h to -80 mV. The majority of the 20 episodes contained no channel openings. **D.** All-point histogram of the events at 0 mV in the presence of PDBu from $V_h = -80$ mV. Fully open channel current was unaffected but NP_o was markedly reduced to 0.185 . **E.** Whole-oocyte currents evoked during application of PDBu. Test depolarization to $+40$ mV. **F.** Time course of decrease of $I_{Kv1.2}$ on application of PDBu $30\ nM$ (arrow). This effect was not readily reversible.

some channel openings, but the majority of test pulses generated blank records (Fig. 6C). An all-points histogram plot of these data revealed that of the channels that did open after PDBu treatment, they opened to the same conductance levels as in control conditions, but their probability of opening was decreased markedly.

Direct effect of Ca^{2+} on unitary K_v channel currents. External divalent cations, including Ca^{2+} , cause marked shifts in the voltage dependence of steady state activation and inactivation of voltage-gated K^+ channel currents (18). The effect of internal divalent cations, however, varies. Magnesium ions induce inward rectification in A-type K^+ recorded in neurons (29) and in a delayed rectifier K^+ current in smooth muscle cells from the renal artery (30). Ca^{2+} has also been reported to directly decrease K^+ channel currents, albeit at abnormally high intracellular concentrations (10^{-5}

M; Ref. 31). In oocytes expressing m3 receptors, stimulation with ACh raises the [Ca²⁺] in the cytoplasm transiently to a peak of ~300 nM (32). In four excised patches containing K_v1.2 channels, we tested the possibility that Ca²⁺ in the range of 10⁻⁸ to 10⁻⁶ M may contribute to the reduction in I_{Kv} by directly blocking the channel in the same manner as intracellular Mg²⁺. One such experiment is illustrated in Fig. 7. At 10⁻⁸ M [Ca²⁺]_{free}, K_v1.2 channels appeared to open predominantly to the fully open level (Fig. 7, A and B). In the presence of 10⁻⁶ M [Ca²⁺]_{free} (Fig. 7C), channels continued to open in response to depolarizing pulses with approximately the same frequency, but the all-points histogram plot revealed that the majority of openings were made to the smallest subconductance level (Fig. 7D). Despite the increase in the overall probability of opening to any one conductance level (NP_o) from 0.77 to 1.049, there was a decrease in the ensemble-averaged current that could be explained by a decrease in the proportion of openings to the fully open state. These data suggest that Ca²⁺ may directly modulate K_v channels at concentrations approaching 1 μM by altering conductance properties. At [Ca²⁺]_{free} approaching 10⁻⁵ M, unitary channel activity was characterized by rapid flicker-type opening and closings (data not shown).

Effect of ACh on COS-7 cells cotransfected with hm3 and K_v1.5. The hm3 receptor and K_v1.5 channel were also

stably coexpressed in COS cells. Whole-cell I_{Kv1.5} activated at potentials positive to -40 mV and was indistinguishable from macroscopic I_{Kv1.5} recorded in *Xenopus* oocytes. The density of channels expressed in COS cells was ~10-fold higher than in oocytes expressing K_v1.5 channels. Based on a CSA of 0.25 × 10⁻⁴ cm² in COS cells and assuming a unitary current of 0.5 pA at 0 mV (11), the density of functional channels per COS cell was ~3.2 × 10⁸ cm⁻². In oocytes, assuming a CSA of 0.2 cm², dividing the mean whole-oocyte current at 0 mV (Fig. 2B) by the unitary current yields a channel density of 2 × 10⁷ cm⁻². We tested the action of ACh on I_{Kv1.5} in six COS cells. ACh (100 μM) decreased I_{Kv1.5} but by only 25–30% (Fig. 8, A and B). As in oocytes, the time course of suppression was slow (Fig. 8C) and not voltage dependent (Fig. 8D). This reduction was observed in four cells using 5 mM BAPTA-containing pipettes and in two cells using amphotericin B-perforated patches, suggesting that chelation of intracellular Ca²⁺ does not prevent the suppression of current. Furthermore, this suppression was not reversible up to 30 min after washout of ACh. The ACh-mediated decrease in I_{Kv1.5} was sensitive to pretreatment with atropine (1 μM) (two). These data indicate that the m3 receptor-activated second messenger pathway mediating suppression of I_{Kv} in COS cells may be different from that in oocytes.

Discussion

Our findings in both *Xenopus* oocyte and COS cell expression systems demonstrate that m3 receptor stimulation suppresses I_{Kdel}. We infer from these data that muscarinic receptor-mediated depolarization in colonic smooth muscle may be attributed, in part, to suppression of I_{Kdel}. In view of the relatively slow-onset of the m3 receptor-mediated suppression of I_{Kv} compared with the rapid onset and reversibility of the ACh-activated cation current (19), muscarinic receptor-mediated suppression of I_{Kdel} in the smooth muscle may form the basis of a long-term regulation of membrane excitability. Phorbol esters strongly mimicked the action of m3 receptor stimulation, suggesting that suppression of K_v channel currents follows activation of PKC. Moreover, phorbol esters have been shown to stimulate phosphorylation of K_v1 channels on a time scale similar to suppression of I_{Kv} (33). A consensus sequence for phosphorylation of a serine residue by PKC is found in all Shaker K_v1 channels in the intracellular loop between transmembrane segments S4 and S5 (31). Our data, like that of Huang *et al.* (33), suggest that the point of convergence between the actions of ACh and phorbol esters may be PKC. We found that staurosporine appeared to be more effective at inhibiting the actions of phorbol esters on I_{Kv} than the suppression of I_{Kv} through m3 receptor stimulation. H7, however, attenuated the m3 receptor-mediated suppression of I_{Kv} but was less effective than m3 receptor blockade with atropine. The partial but not complete inhibition of the m3 receptor-mediated responses on I_{Kv} suggest that there may be mechanisms other than activation of PKC that result in suppression of I_{Kv}. Components of the pathway linking m3 receptors to ion channels include IP₃, DAG, and Ca²⁺ (32). IP₃ triggers the release of Ca²⁺ from stores, whereas DAG binds to the regulatory site of PKC and activates it (34), but IP₃ alone has little effect on ion channel activity. Ca²⁺ alone, at suprabasal levels, however, may directly modulate K⁺ channel activity (35). How-

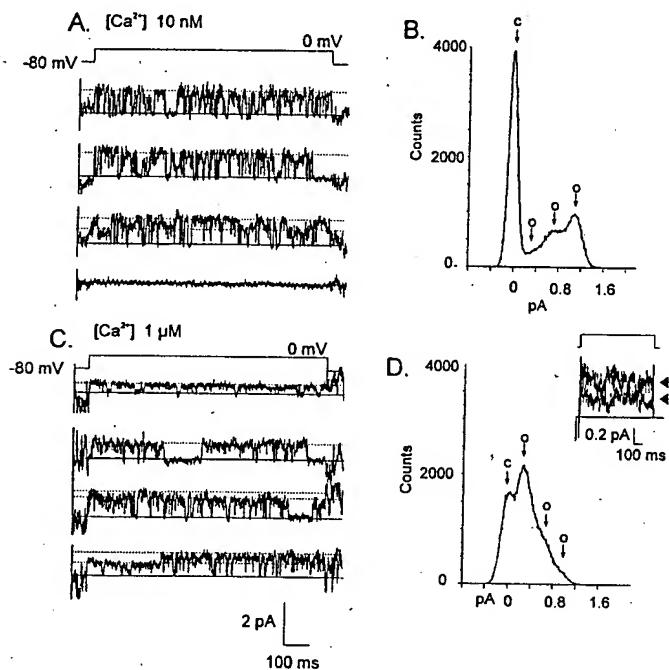


Fig. 7. Effect of increase in [Ca²⁺]_{free} on K_v1.2 single-channel currents in inside-out patch. A, Cytoplasmic surface of the patch was exposed to a high-K (100 mM) solution containing 10 nM [Ca²⁺]_{free}, titrated with EGTA. Patch depolarization to 0 mV from a holding potential of -80 mV elicited opening of a single channel. Four consecutive episodes are shown. B, All-points histogram of 16 consecutive episodes as shown in A. Note presence of three open states (O). C, [Ca²⁺]_{free} was increased to 1 μM. Channel openings again were elicited during the majority of pulses to 0 mV, from a holding potential of -80 mV. However, the frequency of openings to the subconductance states appeared to increase. D, All-points histogram of evoked events in 1 μM [Ca²⁺]_{free}. The current levels representing open states were unchanged, but the proportion of openings to the smallest conductance level was increased. Overall NP_o was increased from 0.77 in 10 nM [Ca²⁺]_{free} to 1.049 in 1 μM [Ca²⁺]_{free}, but the ensemble-averaged current was decreased in the elevated [Ca²⁺]_{free}.

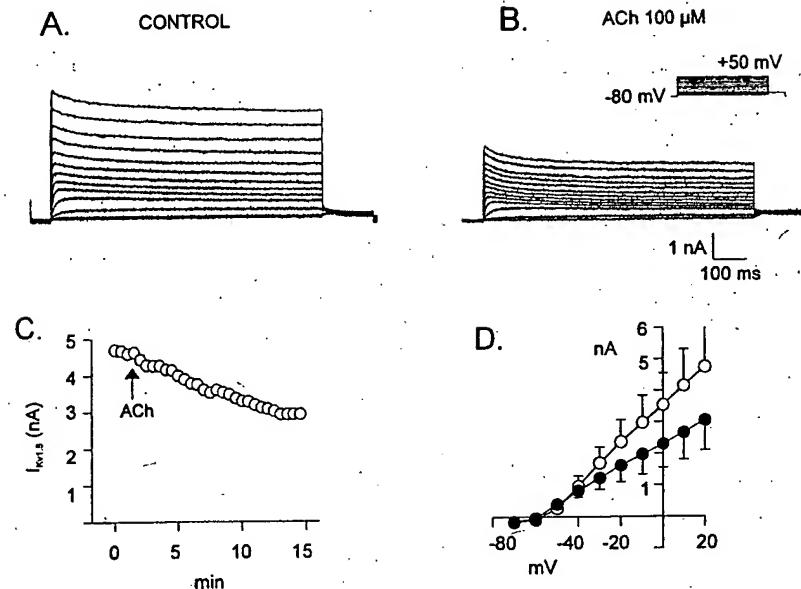


Fig. 8. Effect of ACh on whole-cell $I_{Kv1.5}$ recorded from stably transfected COS cells cotransfected with hm3 receptor. **A.** Control currents. Cell was depolarized from a holding potential of -80 mV to test potentials ranging from -70 to $+50$ mV in 10 -mV increments every 8 sec. **B.** Currents elicited in the same cell in response to the same stimuli as in **A** after the application of ACh (100 μ M). **C.** Time course of suppression of $I_{Kv1.5}$ in response to ACh application (arrow). **D.** Mean I-V relationship of $I_{Kv1.5}$ before (○) and after (●) application of ACh (four).

ever, previous studies on related channels have ruled out Ca^{2+} having direct inhibitory actions on K_v channels at physiological concentrations (31). In excised patches containing $K_v1.2$ channels, we observed that at $[\text{Ca}^{2+}]_{\text{cyt}}$ approaching 1 μM the frequency of channel openings to subconductance states was increased compared with the channel openings elicited in $[\text{Ca}^{2+}]_{\text{cyt}}$ of 10 nM. This switching in preferred channel conductance caused a net reduction in the ensemble current (Fig. 8D, inset). This effect was reversible within tens of seconds, unlike the action of Ca^{2+} ionophores and ACh on I_{Kv} , which recovered only partially after perfusion with ACh-free and Ca^{2+} -free solution for up to 40 min. In *Xenopus* oocytes, elevation in $[\text{Ca}^{2+}]_{\text{cyt}}$ after m3 receptor stimulation rarely exceeds 300 nM and returns to basal levels within 20 sec (32). However, if $[\text{Ca}^{2+}]$ in the submembrane space exceeds the level in the cytoplasm at the peak of the response to m3 receptor stimulation, then a direct effect on K_v channel activity cannot be ruled out. However, this effect would be transient and is unlikely to contribute to the sustained suppression of I_{Kv} . At the level of the single channel, ACh and PDBu appeared to decrease the open probability without affecting conductance (see Figs. 5 and 6), making it unlikely that the suppression of I_{Kv} in response to m3 receptor stimulation was mediated in its entirety by a direct effect of Ca^{2+} on the channels. Ca^{2+} released from IP_3 -sensitive stores may also act on Ca^{2+} -dependent isoforms of PLC. *Xenopus* oocytes express an isoform of PLC that closely resembles the mammalian $\text{PLC}_{\beta 1}$ isoform and is activated by a PTX-sensitive G_o protein (26). In oocytes, G_o is coupled to a number of receptors, including m1. The m3 receptor-mediated suppression of I_{Kv} was inhibited by PTX, suggesting that in oocytes G_o rather than G_q couples the m3 receptor to the *Xenopus*-specific isoform of PLC (36). It is not known whether this *Xenopus*-specific PLC is also Ca^{2+} sensitive. However, such isoforms of PLC (23) in oocytes cannot be ruled out (37). We speculate that if this isoform is expressed in oocytes, then elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ from IP_3 -sensitive stores may feedback on PLC and reinforce the hydrolysis of PIP_2 . The net effect would be enhanced production of DAG and increased stimulation of PKC.

An important potential mediator that has been overlooked

in the physiological actions of agonists that activate the PLC pathway is DAG. In rat sympathetic neurons (38) and toad gastric smooth muscle (39), DAG generated from either PIP_2 through the action of PLC has been proposed as the diffusible second messenger responsible for inhibiting M-current. In the present study, both the Ca^{2+} dependence and the PTX sensitivity of the m3 receptor-mediated suppression of I_{Kv} may be equally well explained in terms of activation of a Ca^{2+} -sensitive PLC isoform that, in oocytes, is coupled to the m3 receptor through G_o . This interpretation is supported by mimicry of the inhibitory action of ACh by phorbol esters, which are potent analogues of DAG. More recently, Bowlby and Levitan (40) reported that direct application of DOG (a DAG analogue) to inside-out patches reversibly blocked K_v1 channel currents. In the present study using cell-attached patches, we demonstrated that the putative second messenger can diffuse to a region of membrane unstimulated by ACh and inhibit the opening of $K_v1.2$ channels.

In the present study, we demonstrated that m3 receptor stimulation causes marked suppression of voltage-gated K^+ channel currents generated by two delayed rectifier K^+ channels cloned from canine colonic smooth muscle. We conclude that muscarinic receptor-mediated excitation in colonic smooth muscle may be partly attributed to suppression of I_{Kdcl} generated by heteromeric $K_v1.2/K_v1.5$ channels. *In situ* experiments are now appropriate and necessary to corroborate these molecular experiments and further delineate the role of these delayed rectifier K^+ channels in colonic mechanical activity.

Acknowledgments

The authors thank Ernest Peralta for hm3 cDNA. The authors also thank Nancy Horowitz for transfection and maintenance of COS cells and Kathleen Keef for critical reading of the manuscript.

References

- Levitin, I. B. Modulation of ion channels in neurons and other cells. *Annu. Rev. Neurosci.* 11:119–136 (1988).
- Payet, M. D., and G. Dupuis. Dual regulation of the *n* type K^+ channel in Jurkat T lymphocytes by protein kinase A and *C. J. Biol. Chem.* 267: 18270–18273 (1992).
- Selyanko, A. A., C. E. Stansfeld, and D. A. Brown. Closure of potassium M

- channels by muscarinic acetylcholine-receptor stimulants requires a diffusible messenger. *Proc. R. Soc. Lond. B Biol. Sci.* 250:119–125 (1992).
4. Caulfield, M. P., S. Jones, Y. Vallis, N. J. Buckley, G.-D. Kim, G. Milligan, and D. A. Brown. Muscarinic M-current inhibition via G_{αq/11} and α-adrenoceptor inhibition of Ca²⁺ current via G_{αo} in rat sympathetic neurones. *J. Physiol. (Lond.)* 474:415–422 (1994).
 5. Bosma, M. M., and B. Hille. Protein kinase C is not necessary for peptide-induced suppression of M current or for desensitization of the peptide receptors. *Proc. Natl. Acad. Sci. USA* 86:2943–2947 (1989).
 6. Beech, D. J., L. Berneheim, A. Mathie, and B. Hille. Ca²⁺ buffers disrupt muscarinic suppression of Ca²⁺ current and M current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. USA* 88:652–656 (1991).
 7. Clapp, L. H., A. M. Sims, J. J. Singer, and J. V. Walsh. Role for diacylglycerol in mediating the actions of ACh on M-current in gastric smooth muscle cells. *Am. J. Physiol.* 263:C1274–C1281 (1992).
 8. Ffrench-Mullen, J. M. H., C. R. Plata-Salaman, N. J. Buckley, and P. Danks. Muscarinic modulation by a G protein, a subunit of delayed rectifier K⁺ current in rat ventromedial hypothalamic neurones. *J. Physiol. (Lond.)* 474:21–26 (1994).
 9. Kavanagh, M. P., M. J. Christie, P. B. Osborne, A. E. Busch, K.-Z. Shen, Y. Wu, P. H. Seeburg, J. P. Adelman, and R. A. North. Transmitter regulation of voltage-dependent K⁺ channels expressed in *Xenopus* oocytes. *Biochem. J.* 277:899–902 (1991).
 10. Hart, P. J., K. E. Overturf, S. N. Russell, A. Carl, J. R. Hume, K. M. Sanders, and B. Horowitz. Cloning and expression of a K_v1.2 class delayed rectifier K⁺ channel from canine colonic smooth muscle. *Proc. Natl. Acad. Sci. USA* 90:9659–9663 (1993).
 11. Overturf, K. E., S. N. Russell, A. Carl, F. Vogalis, P. J. Hart, J. R. Hume, K. M. Sanders, and B. Horowitz. Cloning and characterization of a K_v1.5 delayed rectifier K⁺ channel from vascular and visceral smooth muscles. *Am. J. Physiol.* 267:C1231–C1238 (1994).
 12. Russell, S. N., K. E. Overturf, and B. Horowitz. Heterotetramer formation and charybdotoxin sensitivity of two K⁺ channels cloned from smooth muscle. *Am. J. Physiol.* 267:C1729–C1733 (1994).
 13. Carl, A. Multiple components of delayed rectifier K⁺ current in canine colonic myocytes. *J. Physiol. (Lond.)* 484:339–353 (1995).
 14. Thornbury, K. D., S. M. Ward, and K. M. Sanders. Participation of fast-activating, voltage-dependent K currents in electrical slow waves of colonic circular muscle. *Am. J. Physiol.* 263:C226–C236 (1992).
 15. Keef, K. D., S. M. Ward, R. J. Stevens, B. W. Frey, and K. M. Sanders. Electrical and mechanical effects of acetylcholine and substance P in subregions of canine colon. *Am. J. Physiol.* 262:G298–G307 (1992).
 16. Zhang, L. B., B. Horowitz, and I. L. Buxton. Muscarinic receptors in canine colonic circular smooth muscle: I. Coexistence of M2 and M3 subtypes. *Mol. Pharmacol.* 40:943–951 (1991).
 17. Lechleiter, J., R. Hellmiss, K. Duerson, D. Ennulat, N. David, D. Clapham, and E. Peralta. Distinct sequence elements control the specificity of G protein activation by muscarinic acetylcholine receptor subtypes. *EMBO J.* 9:4381–4390 (1990).
 18. Mayer, M. L., and K. Sugiyama. A modulatory action of divalent cations on transient outward current in cultured rat sensory neurones. *J. Physiol. (Lond.)* 396:417–433 (1988).
 19. Lee, H. K., O. Bayguinov, and K. M. Sanders. Role of nonselective cation current in muscarinic responses of canine colonic muscle. *Am. J. Physiol.* 265:C1463–C1471 (1993).
 20. Hamill, O. P., A. Marty, B. Neher, and F. J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391:85–100 (1981).
 21. Rae, J., K. Cooper, G. Gates, and M. Watsky. Low access resistance perforated patch recordings using amphotericin B. *J. Neurosci. Methods* 37:15–21 (1991).
 22. Peralta, E. G., A. Ashkenazi, J. W. Winslow, D. H. Smith, J. Ramachandran, and D. J. Capon. Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J.* 6:3923–3929 (1987).
 23. Berridge, M. J. Inositol triphosphate and calcium signalling. *Nature (Lond.)* 361:315–325 (1993).
 24. Sahara, S., K. Sato, M. Aoto, T. Ohnishi, H. Kaise, H. Koide, K. Ogita, and Y. Fukami. Characterization of protein kinase C in *Xenopus* oocytes. *Biochem. Biophys. Res. Commun.* 182:105–114 (1992).
 25. Ashkenazi, A., E. G. Peralta, J. W. Winslow, J. Ramachandran, and D. J. Capon. Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. *Cell* 56:487–493 (1989).
 26. Ma, H., R. D. Blitzer, E. C. Healy, R. T. Premont, E. M. Landau, and R. Iyengar. Receptor-evoked Cl⁻ current in *Xenopus* oocytes is mediated through a β-type phospholipase C. *J. Biol. Chem.* 268:19915–19918 (1993).
 27. Tobin, A. B., B. Keys, and S. R. Nahorski. Phosphorylation of a phosphoinositidase C-linked muscarinic receptor by a novel kinase distinct from b-adrenergic receptor kinase. *FEBS Lett.* 335:353–357 (1993).
 28. Murray, K. T., S. A. Fahrig, K. K. Deal, S. S. Po, N. N. Hu, D. J. Snyders, M. M. Tamkun, and P. B. Bennett. Modulation of an inactivating human cardiac K⁺ channel by protein kinase C. *Circ. Res.* 75:999–1005 (1994).
 29. Forsythe, I. D., P. Linsdell, and P. R. Stanfield. Unitary A-currents of rat locus coeruleus neurones grown in cell culture: rectification caused by internal Mg²⁺ and Na⁺. *J. Physiol. (Lond.)* 451:553–583 (1992).
 30. Gelband, C. H., T. Ishikawa, J. M. Post, K. D. Keef, and J. R. Hume. Intracellular divalent cations block smooth muscle K⁺ channels. *Circ. Res.* 73:24–34 (1993).
 31. Aiyar, J., S. Grissmer, and K. G. Chandy. Full-length and truncated Kv1.3 K⁺ channels are modulated by 5-HT_{1c} receptor activation and independently by PKC. *Am. J. Physiol.* 265:C1571–C1578 (1993).
 32. Lechleiter, J. D., and D. E. Clapham. Molecular mechanisms of intracellular calcium excitability in *X. laevis* oocytes. *Cell* 69:283–294 (1992).
 33. Huang, X. Y., A. D. Morielli, and E. G. Peralta. Tyrosine kinase-dependent suppression of a potassium channel by the G protein-coupled m1 muscarinic acetylcholine receptor. *Cell* 75:1145–1156 (1993).
 34. Bell, R. M., and D. J. Burns. Lipid activation of protein kinase C. *J. Biol. Chem.* 266:4661–4664 (1991).
 35. Gelband, C. H., and J. R. Hume. [Ca²⁺]_i inhibition of K⁺ channels in canine renal artery: novel mechanism for agonist-induced membrane depolarization. *Circ. Res.* 77:121–130 (1995).
 36. Cockcroft, S., and G. M. H. Thomas. Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochem. J.* 288:1–14 (1993).
 37. Blitzer, R. D., G. Ömri, M. De Vivo, D. J. Carty, R. T. Premont, J. Codina, L. Birnbaumer, S. Cotecchia, M. G. Caron, R. J. Lefkowitz, E. M. Landau, and R. Iyengar. Coupling of the expressed α_{1B}-adrenergic receptor to the phospholipase C pathway in *Xenopus* oocytes: the role of G_o. *J. Biol. Chem.* 268:7532–7537 (1993).
 38. Brown, D. A., N. V. Marion, and T. G. Smart. On the transduction mechanism for muscarine-induced inhibition of M-current in cultured rat sympathetic neurones. *J. Physiol. (Lond.)* 413:469–488 (1989).
 39. Sims, S. M. Calcium and potassium currents in canine gastric smooth muscle cells. *Am. J. Physiol.* 262:G859–G867 (1992).
 40. Bowley, M. R., and I. B. Levitan. Block of cloned voltage-gated potassium channels by the second messenger diacylglycerol independent of protein kinase C. *J. Neurophysiol.* 73:2221–2229 (1995).

Send reprint requests to: Dr. Burton Horowitz, Department of Physiology, University of Nevada, School of Medicine, Reno, NV 89557-0046.